REVIEW

The role of apoptosis-inducing receptors of the tumor necrosis factor family in thyroid cancer

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Abstract

The tumor necrosis factor (TNF) family comprises several ligands, such as the prototype TNF-α, the Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL/Apo2L), which trigger apoptosis in susceptible cells by activating respective cell-surface receptors. The study of these cell death pathways has attracted significant attention in several fields, including that of thyroid cancer, because they participate in immune system function, as an arm of cell-mediated cytotoxicity, and because recombinant ligands are available for pharmacological use. TNF-α is a pluripotent cytokine that induces both pro-apoptotic and anti-apoptotic effects on thyroid carcinoma cells. FasL triggers apoptosis in other tumor types, but thyroid carcinoma cells are resistant to this effect. On the other hand, TRAIL potently and selectively kills thyroid carcinoma cells. Consequently, TRAIL is the only member of the family with significant anticancer activity and an acceptable toxicity profile to be used as a novel therapy for thyroid cancer. The caspase inhibitor FLIP plays a significant role in negatively regulating receptor-induced apoptosis. Thelper 1-type cytokines, such as interferon-γ, TNF-α and interleukin-1β increase the sensitivity of both normal and neoplastic thyrocytes to FasL and TRAIL. On the other hand, IGF-I and other growth/survival factors produced in the local tumor microenvironment activate the phosphatidylinositol 3-kinase/Akt kinase pathway and exert an anti-apoptotic effect by upregulating several apoptosis inhibitors, including FLIP. Pharmacological modulation of apoptosis induced by FasL and TRAIL/Apo2L holds promise of therapeutic applications in human malignancies.

Introduction

In recent years, a surge in our knowledge about programmed cell death (apoptosis) has established its role in the regulation of tumorigenesis. Apoptosis is an active, energy-dependent process that mediates the elimination of cells that have developed improperly, or have sustained irreparable genetic damage (Thompson 1995). Therefore, inadequate cell death contributes, along with increased cell proliferation, to the emergence of hyperplasias and neoplasias (Thompson 1995, Zhivotovsky & Orrenius 2003). Many oncogenes that participate in human cancer pathophysiology, such as ras and Bcl-2, represent overactive or overexpressed versions of proto-oncogenes that normally function by negatively regulating cell death. Moreover, several tumor suppressor genes that are frequently mutated or deleted in human cancers are, in their native form, inducers of apoptosis that safeguard the body from the emergence of neoplastic cells. A classic example is the ‘guardian of the genome’ p53, which, upon induction of DNA damage, is pivotal in the DNA repair process and, if the latter is not possible, triggers the apoptotic elimination of the damaged cell in order to avoid the emergence of mutant progeny (Lebedeva et al. 2003). p53 is inactivated in approximately 50% of human cancers, representing the most common genetic aberration in human malignancy. Anti-apoptotic molecules, such as Bcl-2 and Bcl-xL, play pathogenetic roles not only in the emergence of various human malignancies, but contribute to their resistance to anticancer chemotherapy as well (Adams & Cory 1998, Coultas & Strasser 2003).

The apoptotic mechanism is triggered by intracellular as well as extracellular stimuli. Developmental cell death, which occurs during several stages of fetal development, is crucial for the natural involution of cells and structures that are no longer necessary for the embryo’s development (Haanen & Vermes 1996). In the cancer treatment setting, ionizing radiation and several chemotherapeutic agents...
induce DNA damage that subsequently triggers an intracellular signaling pathway that results in apoptosis (Thompson 1995, Zhivotovsky & Orrenius 2003). Extracellular pro-apoptotic stimuli could be transduced by the stimulation (or, in other cases, the lack of stimulation) of cell-surface or nuclear receptors. For example, lack of growth/survival factors, frequently simulated in vitro by serum starvation, results in the activation of an intrinsic cell death pathway that is normally suppressed in the presence of paracrine stimulation (Mitsiades et al. 2002). Androgen withdrawal, commonly effected in vivo by surgical or chemical castration, triggers the programmed cell death pathway in both normal prostate glandular epithelia and androgen-dependent prostate cancer cells (Denmeade et al. 1996). In recent years, intense study has focused on the apoptosis signaling pathways triggered, via respective cell-surface receptors, by Fas ligand (FasL) and tumor necrosis factor (TNF)–related apoptosis–inducing ligand (TRAIL)/Apoptosis–inducing ligand (TRAIL)/Apoptosis inhibitor of cell death (Drzetic et al. 2003), members of the TNF family of death ligands (Ozoren & El-Deiry 2003). The present review will provide a background on the major players of the TNF family, will discuss their role in the pathogenesis of thyroid cancer and will present an update on the ongoing efforts to evaluate them as a potential anticancer therapeutic agent.

The important role of the Fas and TRAIL apoptotic pathways in thyroid autoimmunity (Hashimoto’s and Graves’ disease) will not be specifically addressed in this review. For a detailed analysis of that subject, the reader is directed to previous reviews (Andrikoula & Tsatsoulis 2001, Mitsiades et al. 2001c, Stassi & De Maria 2002, Tsatsoulis 2002).

**Background**

The TNF family comprises a growing number of extracellular ligands (CD40L, CD137L/4–1 BBL, CD134L/OX40L, CD27L/CD70, CD30L, TNF–β/LT–α, LT–β, TWEAK/Apo3L, TNF–α, FasL, TRAIL) with complex, diverse and often overlapping roles in B and T cell development, lymphocyte activation, cytokine production, humoral immune response, cell-mediated cytotoxicity and apoptosis (Gruss 1996). Most are expressed on a broad spectrum of normal hematopoietic cells (especially after activation), as well as on many neoplastic cell lines (Gruss 1996, Vinay & Kwon 1998). TNF–α, FasL and TRAIL are the ligands most frequently involved in apoptotic signaling and are synthesized as type II transmembrane proteins (i.e. their amino–terminal end is extracellular) that belong in the TNF/nerve growth factor receptor superfamily. They harbor cysteine-rich extracellular domains and a cytoplasmic domain of approximately 70 amino acid residues which is both necessary and sufficient for the induction of apoptosis (the death domain, DD) (Schulze-Osthoff et al. 1998). Upon cross-linking by its respective ligand, the receptor is oligomerized and recruits via its DD an adapter molecule. The amino-terminal region of the adapter molecule, named the death effector domain (DED) then recruits another DED-containing molecule, the pro–enzyme form of caspase–8 or caspase–10, also known as FLICE (Fas–associating protein with a DD (FADD)–like interleukin–1 beta–converting enzyme) and FLICE–2 respectively, resulting in proteolytic autoactivation of the apical caspase (induced–proximity model) (Muzio et al. 1998, Salvesen & Dixit 1999), which then transmits the apoptotic signal via the downstream apoptotic caspase cascade. This apoptotic cascade has been called ‘instructive apoptosis’ (Ashkenazi & Dixit 1998, 1999) because it serves as a paracrine mechanism that enables a multicellular organism to instruct individual cells to die. This signaling pathway has a very high degree of specificity and is particularly important for the function of the immune system (Ashkenazi & Dixit 1998, 1999).

**TNF–α and its receptors**

Human TNF–α exists as a 26 kDa biologically active transmembrane protein expressed on the surface of a wide variety of hematopoietic and non-hematopoietic cells, and as a 17 kDa, also biologically active, soluble cytokine that is generated by proteolytic cleavage by TNF–α–converting enzyme and circulates as a homotrimer (Gruss 1996, Moss et al. 1997). TNF–α signals through two different receptors, TNFRI/p55/CD120a and TNFRII/p75/CD120b, which are expressed in most cells. TNFRI is a 55 kDa transmembrane glycoprotein, with an extracellular region harboring four cysteine-rich motifs and a cytoplasmic domain containing a DD. Upon cross-linking, TNFRI binds the adapter molecule TNFR–associated DD, which promotes the recruitment and activation of pro–caspase–8, thus triggering the apoptotic cascade (Ashkenazi & Dixit 1998). TNFRI can also trigger anti–apoptotic and activation pathways, namely the activation of the transcription factor NF–kB via the kinases receptor–interacting protein and NF–kB–inducing kinase, and the transcription factor AP–1, via recruitment of the TRAF2 (TNF–associated factor–2) and activation of the MEKK1/JNK/JNK pathway (Ashkenazi & Dixit 1998). The role of the 75 kDa TNFRII is still debated. It has been suggested that TNFRII binds TNF–α and transfers it to TNFR, which then is activated and initiates its own signaling cascade (Tartaglia et al. 1993). However, direct
TNFRII-mediated TNF-α signaling has also been demonstrated (Jupp et al. 2001). Moreover, by recruiting TRAF2 and inhibitors of apoptosis proteins (IAPs) that bind to it, TNFRII can prevent these anti-apoptotic proteins from interfering with the pro-apoptotic function of TNFRI and may accelerate TNFRI-dependent activation of caspase-8 (Fotin-Melczek et al. 2002).

TNF-α is considered to play a pivotal role in the pathophysiology of a wide spectrum of immune-mediated conditions, such as shock and rheumatoid arthritis. Systemic administration of TNF-α results in a clinical picture that mimicks that of shock, and antibodies to TNF protect animals against the deleterious effects of i.v. injections of either lipopolysaccharide or live bacteria (Simpson & Casey 1989, Tracey 1991). Two TNF-α antagonists have been approved for the treatment of rheumatoid arthritis by the United States Food and Drug Administration (FDA): a soluble p75 TNF-α receptor fusion protein, consisting of a genetic fusion of recombinant soluble p75 TNF-α receptor to the Fc portion of IgG (Etanercept) (Weinblatt et al. 1999), and an anti-TNF-α antibody (Infliximab) (Lipsky et al. 2000). Infliximab has also been FDA approved for the treatment of Crohn’s disease (Present et al. 1999).

Fas and FasL

Fas/Ap1/CD95 is widely expressed in normal and neoplastic tissues (Schulze-Osthoﬀ et al. 1998). Upon cross-linking (by FasL or polyvalent antibodies), its cytoplasmic DD recruits the adapter FADD, also known as MORT1 (Schulze-Osthoﬀ et al. 1998), which, via its amino-terminal DED, then recruits the pro-enzyme form of caspase-8 (Schulze-Osthoﬀ et al. 1998). The aggregation of Fas, FADD and caspase-8, named the death-inducing signaling complex (DISC), catalyses the proteolytic autoactivation of caspase-8 (induced-proximity model) (Muzio et al. 1998, Salvesen & Dixit 1999), which then activates the downstream caspase cascade.

Fas is expressed in activated T lymphocytes, where it participates in cell-mediated cytotoxicity against virally infected or transformed cells, as well as in the testis, the anterior chamber of the eye, the placenta and the brain (Schulze-Osthoﬀ et al. 1998), where it contributes to the immune-privileged status of these organs by eliminating infiltrating lymphocytes. Fas-mediated apoptosis is, therefore, a key effector component of the immune system’s antitumor surveillance function and also contributes to immune homeostasis, by eliminating autoreactive lymphocytic clones and mediating the suicidal elimination of activated immune cells at the end of an inflammatory reaction.

Fas and FasL also participate in the inflammatory destruction of target organs in several autoimmune disorders, including Hashimoto’s thyroiditis (Giordano et al. 1997, Hammond et al. 1997, Arscott & Baker 1998, Mitsiades et al. 1998b, Borgerson et al. 1999). Concurrent strong presence of both FasL and Fas has been detected on the surface of thyrocytes in Hashimoto’s thyroiditis and they co-localize with the presence of apoptotic thyrocytes, with both being inversely proportional to the distance from infiltrating lymphocytes. These findings suggest that the FasL–Fas system is important for a suicidal/fratricidal apoptotic process in this setting and hence, in the pathogenesis of Hashimoto’s thyroiditis. The infiltrating lymphocytes themselves express little to no FasL, implying that they are not directly engaged in the killing of thyrocytes with their own FasL (Mitsiades et al. 1998b). This finding agrees with earlier studies that determined that infiltrating activated T lymphocytes in Hashimoto’s thyroiditis do not have direct cytotoxic potential, but rather induce thyrocyte apoptosis via production of cytokines (Weetman & McGregor 1994). Lymphocyte-derived Thelper 1-type cytokines, specifically interferon (IFN)-γ, TNF-α and interleukin (IL)-1β, can sensitize thyrocytes to Fas-mediated apoptosis (Bretz et al. 1999a), by upregulating Fas (Bretz et al. 1999a) and caspase-8 and -3 (Stassi et al. 2000). Moreover, expression of the apoptosis inhibitor Bcl-2 is downregulated in thyrocytes in Hashimoto’s thyroiditis (Mitsiades et al. 1998b). Therefore, the follicular destruction in Hashimoto’s thyroiditis appears to result from the imbalance between apoptosis-promoting and –inhibiting molecules.

In regards to the antitumor activity of the Fas pathway, many (if not the majority of) tumor cell lines and primary cells have been reported to be resistant to its apoptotic activity. Several potential mechanisms for resistance to Fas-mediated apoptosis have been described in cancer cells, including downregulation of Fas protein expression, intracytoplasmic sequestration and failure of the receptor to translocate to the cell surface, production and secretion of a soluble form of ‘decoy’ receptor (either an alternatively spliced form of Fas or another soluble inhibitor of Fas activation (decoy receptor 3, DcR3)), or mutations of Fas, especially in the DD (Muschen et al. 2000). Obviously, different mechanisms may be operative in different patients and histological types of malignancies. A more widespread mechanism of resistance to Fas-mediated apoptosis is the overexpression of anti-apoptotic proteins. FLIP is an inhibitor of the Fas signaling pathway with a structure similar to caspase-8 (Irmler et al. 1997) that binds to FADD, yet is catalytically inactive and interferes with the proper formation of the DISC complex, thus representing a naturally occurring dominant negative form of caspase-8 (Fig. 1). Another Fas-inhibitory protein, Fas-associated phosphatase-1, which binds to the 15 carboxy-terminal amino acids of the receptor, has been associated with resistance to Fas-mediated apoptosis (Mye et al. 1999), at least in some models.

Not only have many cancer cells become resistant to apoptosis induced through Fas, but, in many cases, they have acquired the ability to utilize this pathway to their advantage, by launching a ‘Fas counterattack’ against
the host’s immune system. In particular, FasL has been detected in a wide range of neoplasms, and, since activated lymphocytes are sensitive to Fas-mediated apoptosis, it has been postulated that it induces apoptosis of tumor-infiltrating immune cells (O’Connell et al. 1996, Gratas et al. 1997, 1998, Niehans et al. 1997, Mitsuodes et al. 1998a,b). FasL-expressing tumor cell lines have been found to induce apoptosis in Fas-sensitive cells of lymphocytic origin in vitro, while apoptotic immune cells are present in the vicinity of Fas-positive neoplastic cells in vivo (Hahne et al. 1996). In support of a functional role for FasL in cancer progression and immune evasion in vivo, strong FasL expression correlated with increased aggressiveness and metastatic potential in several histological types of cancer (Mitsuodes et al. 1998a). In most cases, this ‘ectopic’ expression of FasL by tumor cells coincides with their inherent resistance to Fas-mediated apoptosis, which protects them from a ‘suicidal’ death. Yet, there are cases where Fas-sensitive tumor cells produce their own FasL and yet avoid a Fas–FasL–mediated suicide because they cleave FasL into a soluble form through the action of a metalloproteinase and avoid its accumulation on their cell surface (Mitsuodes et al. 2001b,d).

**TRAIL/Apo2L and its receptors**

TRAIL/Apo2L is a 32 kDa transmembrane protein expressed on a wide range of normal fetal and adult tissues, suggesting the existence of a protective mechanism against its cytotoxicity in normal cells. This is supported by observations that TRAIL/Apo2L can induce apoptosis in transformed and malignant cells, but not in normal cells (Ashkenazi & Dixit 1999). TRAIL induces apoptosis by interacting with two cell-surface receptors, DR4 (or TRAIL–R1) and DR5 (or TRAIL–R2) (Ashkenazi & Dixit 1999). Three additional receptors for TRAIL, which cannot transduce an apoptotic signal, were also cloned: TRAIL–R3 (TRID, DcR1), TRAIL–R4 (TRUNDD, DcR2) and osteoprotegerin (Ashkenazi & Dixit 1999).

Initially, the presence of the mRNAs for these ‘decoy’ receptors was reported in non-neoplastic cells only, and it was proposed to be the reason for their resistance to TRAIL/Apo2L. However, subsequent studies revealed the presence of ‘decoy’ receptor mRNA and protein in cancer cells as well (Mitsiades et al. 2000, 2001a,b). Thus, the mechanism of resistance of normal cells to TRAIL/Apo2L, as well as the functional significance of the ‘decoy’ receptors, are still elusive.

The selective nature of the anticancer activity of TRAIL was demonstrated with studies in mice and non-human primates (Ashkenazi et al. 1999, Walczak et al. 1999). However, subsequent reports have cautioned that recombinant TRAIL/Apo2L may kill normal human hepatocytes in vitro (Jo et al. 2000, Ozoren et al. 2000). It has been suggested that this finding was due to non-optimized recombinant ligand preparations (Lawrence et al. 2001). Yet, clinical development of recombinant TRAIL has been delayed pending further studies. TRAIL-mimicking antibody-1, which binds to and activates TRAIL–R1, is currently being evaluated in phase I clinical trials in solid and hematological malignancies (Salcido et al. 2002).

The activity of death ligands against thyroid carcinomas

**TNF-α**

TNF-α is produced locally by lymphocytes in areas of thyroid inflammation (Aust et al. 1996) and TNF-α receptors are expressed on thyroid carcinoma cells (Pang et al. 1989). Contrary to IFN-γ, which is consistently...
anti-proliferative and pro-apoptotic (Ohta et al. 1996), TNF-α has been reported to exert both pro- and anti-apoptotic effects on thyroid carcinoma cell lines (Ohta et al. 1996, Pang et al. 1996). The anti-apoptotic effects should be attributed to the ability of TNF-α to activate NF-κB, a transcription factor with cytoprotective actions, and to induce enzymes thought to be cytoprotective, such as manganese superoxide dismutase (MnSOD) (Pang et al. 1992). Cells lines resistant to the pro-apoptotic effect of TNF-α have increased basal expression of MnSOD (Pang et al. 1996). Moreover, TNF-α induces the expression of the anti-apoptotic Bcl-2 family member A1, in a time- and dose-dependent manner (Pang et al. 1997). It should be noted that TNF-α exerts on normal and malignant thyrocytes a strong sensitizing effect to FasL- and TRAIL-induced apoptosis (Bretz et al. 1999a,b, 2002, Mitsiades et al. 2000, Poulaki et al. 2002b). This could be attributed to the induction by TNF-α of several death receptors (Fas, DR5) and caspases (-3, -10) in thyrocytes (Poulaki et al. 2002b). Moreover, TNF-α has a strong stimulatory effect on the production of endogenous TRAIL by thyrocytes themselves (Bretz et al. 1999b, Poulaki et al. 2002b), an effect that further promotes apoptosis. TNF-α also reduced vascular endothelial growth factor secretion by anaplastic tumor cells in vitro (Wang et al. 2002). This suggests that TNF-α can inhibit the secretion of pro-angiogenic stimuli from neoplastic cells. It is also well-known that TNF-α directly induces apoptosis of endothelial cells (Maden & Pober 2001). Therefore, although TNF-α alone has limited direct anticancer activity against thyroid carcinoma cell lines in vitro, it exerts anti-angiogenic effects and its combination with FasL and TRAIL provides a strong synergistic antitumor activity.

**FasL**

The issue of Fas expression in normal thyroid tissue has been at the center of a debate since an early report by Giordano et al. (1997), who suggested that Fas was absent in the tissues that they used as controls in their study of Hashimoto's thyroiditis. However, subsequent studies from several groups, using various laboratory techniques, have concluded the opposite, i.e. Fas is present (Leithauser et al. 1993, Tanimoto et al. 1995, Kawakami et al. 1996, Arscott et al. 1997, Mitsiades et al. 1998b, 2000) in normal thyroid follicular cells, even though the latter are resistant to Fas-mediated apoptosis under normal circumstances in vivo (Arscott et al. 1999). It has been suggested that since the 'control' thyroid tissue of the Giordano et al. (1997) study was derived from non-toxic goiters, it should not be considered truly normal (Mitsiades et al. 1998b, 1999b, Baker 1999).

Since Fas-mediated apoptosis is a key mechanism of T cell-mediated cytotoxicity against neoplastic cells and its inhibition contributes to tumor progression and metastasis, resistance to Fas is commonly found in tumor cells. In some cases, resistance is due to reduced cell-surface Fas expression (Hughes et al. 1997, Nambu et al. 1998). Fas expression has been reported to be low in thyroid nodules (Andrikoulas et al. 2001), a finding that agrees with the results inadvertently obtained by Giordano et al. (1997) in their 'control' non-toxic goiter tissue. Thyrocytes from both normal and nodular tissue are resistant to Fas-mediated cell death in vitro, but, even though the former can be sensitized by pro-inflammatory cytokines (see below), the latter often are not, suggesting the existence of additional blocks in their apoptotic pathway (Mezosi et al. 2002).

In the case of thyroid carcinomas, Fas is widely expressed, both in vivo and in vitro (Mitsiades et al. 2000), and may even be upregulated in neoplastic thyroid cells, compared with normal thyrocytes (Arscott et al. 1999). It is unclear what purpose this upregulation serves for the cancer cell, although it certainly does not pose an evolutionary disadvantage, because, similar to normal and goiter-derived thyrocytes, the Fas pathway is blocked in thyroid carcinoma cells. Indeed, cells from thyroid carcinoma lines are resistant to apoptosis induced by Fas cross-linking (Mitsiades et al. 1999b, 2000), similar to their normal counterparts (Arscott et al. 1997), and hardly any apoptotic nuclei are detected among thyroid carcinoma cells in vivo (Basolo et al. 1997, Mitsiades et al. 1999b).

Not only are thyroid carcinoma cells resistant to the apoptosis-inducing activity of FasL, but also they have been able to harness the activity of this pathway to their advantage. Several histological types of malignant tumors, including thyroid carcinomas, express FasL on their cell surface, and utilize it to escape immune surveillance, by inducing apoptosis of infiltrating lymphocytes (Hahne et al. 1996, Saas et al. 1997, Mitsiades et al. 1998a, 1999b, Rabinovich et al. 1998). In thyroid carcinomas, FasL mRNA and protein have been detected by in situ hybridization and immunohistochemistry in patient specimens, and by Western blotting and RT-PCR in thyroid carcinoma cell lines respectively (Mitsiades et al. 1999b). Moreover, cells from thyroid carcinoma lines can kill Fas-sensitive Jurkat cells in a FasL-dependent manner in co-culture (Mitsiades et al. 1999b). In papillary carcinomas in vivo, high levels of FasL expression correlate independently with aggressive histology and unfavorable clinical presentation (Mitsiades et al. 1999b). These observations point to FasL as a cytolytic molecule that contributes to thyroid carcinoma aggressiveness by launching a 'counter-attack' against the host's immune system. The carcinoma cells themselves are not affected by their own FasL, due to their inherent resistance to Fas-mediated apoptosis (Mitsiades et al. 1999b, 2000).

**TRAIL**

The constitutive resistance of thyroid carcinomas to Fas-mediated apoptosis, combined with the systemic toxicity
of Fas activation (Ogasawara et al. 1993), precludes the clinical use of FasL against thyroid carcinomas (Ashkenazi & Dixit 1999). However, the discovery that another death ligand, TRAIL/Apo2L, potently and selectively targets neoplastic cells (Ashkenazi et al. 1999, Waleczak et al. 1999) sparked widespread interest in its use as a novel anticancer therapeutic modality. The apoptosis-inducing TRAIL receptors TRAIL–R1 and TRAIL–R2 are broadly expressed in thyroid carcinomas and thyroid carcinoma cell lines (Mitsiades et al. 2000). Importantly, TRAIL/Apo2L effectively kills most thyroid cell lines tested, including those originating from anaplastic carcinomas (Ahmad & Shi 2000, Mitsiades et al. 2000). Also, the presence of p53 mutations did not impede the anticancer activity of TRAIL (Mitsiades et al. 2000), suggesting that it can still be effective against poorly differentiated cells that frequently harbor defects in the p53 pathway. Therefore, TRAIL/Apo2L is a very promising new agent against thyroid cancer that may overcome resistance to current therapeutic modalities.

Both caspase-8 and the closely related caspase-10 have been implicated as the apical caspases of TRAIL-induced apoptosis in different models (Kischkel et al. 2000, Sprick et al. 2000, Mitsiades et al. 2001b). In thyroid carcinoma cell lines, TRAIL/Apo2L induces rapid recruitment of caspase-10 to the TRAIL receptor(s) and its activation, followed by activation of caspase-8 and -3 (Mitsiades et al. 2000) (Fig. 1). It appears, therefore, that the initial step of the TRAIL apoptotic cascade is tissue-dependent. This finding also indicates that endogenous caspase-8 can be activated in thyroid carcinoma cells by other caspases, thus overriding the apoptotic block that occurs within the Fas pathway.

Intracellular regulation of death receptor signaling in thyroid carcinoma

As mentioned previously, thyroid carcinomas exhibit differential response to stimulation with the various cell-surface death ligands of the TNF family, with TNF-α reported to activate both pro-apoptotic and anti-apoptotic pathways, whereas TRAIL is potently pro-apoptotic and FasL is unable to induce apoptosis. The complete resistance of thyroid carcinoma cell lines to FasL-induced apoptosis is due to the inability of Fas to recruit and activate caspase-8 at the DISC (Fig. 1) (Mitsiades et al. 2000), which is a necessary step for the effective transduction of the apoptotic signal. Further support for the localization of the apoptotic block at a level upstream of caspase-8 is provided by experiments where transfection of a constitutively active caspase-8 construct resulted in apoptosis of papillary carcinoma cells (Mitsiades et al. 2000). Because the protein synthesis inhibitor cycloheximide can sensitize normal thyrocytes (Arscott et al. 1997) and papillary, follicular and anaplastic carcinoma cell lines (Mitsiades et al. 2000) to Fas-mediated cell death, it appears that Fas itself is genetically intact (thus excluding the possibility of mutations) and its function is attenuated by the presence of (a) short-lived inhibitory protein(s). FLIP is a well-known inhibitor of Fas-mediated apoptosis and could play this role, as it is present in thyroid carcinoma cells both in vivo and in vitro and co-immunoprecipitates with Fas (Mitsiades et al. 2000). Moreover, specific downregulation of FLIP, after transfection of an antisense oligonucleotide, sensitizes thyroid carcinoma cell lines to Fas-mediated apoptosis (C S Mitsiades, V Poulaki & N Mitsiades, unpublished observations).

TRAIL, on the other hand, potently triggers apoptosis in carcinoma cell lines originating from the thyroid follicular epithelium, but not in normal thyrocytes (Mitsiades et al. 2000). Specifically, recombinant TRAIL induced apoptosis in eight of eight cell lines originating from papillary carcinomas, and in two of two cell lines originating from anaplastic carcinomas. TRAIL activated caspase-10 at the receptor level and triggered a caspase-mediated apoptotic cascade (Mitsiades et al. 2000). Resistance among cancer cell lines is rare and, when present, can be reversed by cycloheximide, suggesting, again, a role for (a) short-lived apoptosis inhibitor(s). The previously proposed hypothesis that the TRAIL receptors TRAIL–R3 (DcR1) and TRAIL–R4 (DcR2), which cannot transmit a pro-apoptotic signal, serve as ‘decoy’ receptors (Ashkenazi & Dixit 1999), cannot provide an explanation for TRAIL resistance, because both receptors are expressed in normal and neoplastic thyrocytes in vitro and in vivo and their presence does not correlate with resistance to TRAIL (Mitsiades et al. 2000). Thus, the inhibitor(s) of TRAIL signaling must be intracellular. Similar to results in other tissues (Thome et al. 1997), the caspase-8 inhibitor FLIP modulates TRAIL-induced apoptosis in thyroid carcinomas (Poulaki et al. 2002b). Thyroid carcinoma cells selected in vitro for resistance to TRAIL exhibited higher FLIP levels than parental cells. Cycloheximide downregulated FLIP expression and restored TRAIL sensitivity (Poulaki et al. 2002b). Finally, sensitivity to TRAIL in this model was restored in vitro upon downregulation of FLIP by transfection with a specific anti-sense oligonucleotide (Poulaki et al. 2002b).

It is thus possible that FLIP plays an inhibitory role in both FasL- and TRAIL-induced apoptosis in thyroid carcinomas. Since the latter ligand is significantly more effective against thyroid carcinomas than the former, it appears that the threshold for inhibition of apoptosis is lower in the former pathway. For example, low levels of FLIP could suffice to block Fas-induced apoptosis, yet higher levels may be necessary to affect TRAIL/Apo2L signaling. This model could be explained by differential affinity of the inhibitor for the respective ligand–receptor–caspase signaling complexes. It should be noted that, contrary to the Fas pathway, where the FADD
adapter molecule is the unequivocal mediator of DISC formation, it is yet unclear what molecule plays the adapter role for the TRAIL apoptotic pathway in neoplastic thyrocytes. If that, yet unidentified, adapter has lower affinity for FLIP than FADD does, then inhibition of the TRAIL pathway would require higher levels of FLIP than the Fas pathway.

**The role of the local microenvironment**

One of the major advances in our understanding of cancer pathophysiology in recent years is the realization that the behavior of the neoplastic cell is strongly influenced by direct or indirect interactions with other components of the tumor, such as fibroblasts, lymphocytes or endothelial cells. As a result, the behavior of cultured cancer cells under baseline conditions *ex vivo* may not recapitulate all relevant aspects of their *in vivo* phenotype. The role of the local microenvironment in thyroid cancer is striking in cases of papillary carcinomas with extensive lymphocytic infiltrates (‘peri-tumoral thyroiditis’), which are associated with a more favorable prognosis (Baker 1995). Pro-inflammatory Thelper 1-type cytokines produced by the infiltrating lymphocytes (IFN-γ, TNF-α and IL-1β) have a significant effect on tumor cell behavior and sensitivity to apoptosis. These cytokines stimulate the apoptotic pathway at various levels, as they increase the protein levels of Fas (Mitsiades *et al.* 2000), and caspase-8 and -3 (Stassi *et al.* 2000, Poulaki *et al.* 2002b). As a result, they increase sensitivity of neoplastic (and normal) thyrocytes to FasL (Bretz *et al.* 1999a, Mitsiades *et al.* 2000) and TRAIL (Bretz *et al.* 1999b, 2002, Poulaki *et al.* 2002b). Mezosi *et al.* (2002), however, recently reported that this sensitizing effect of Thelper 1 cytokines was not observed in many cases of goiter-derived thyrocytes, raising the possibility of a different mechanism of apoptosis regulation in thyroid goiter.

Contrary to this pro-apoptotic role of infiltrating lymphocytic populations, other cellular components of the local microenvironment produce soluble factors that may exert an anti-apoptotic effect. For example, in thyroid neoplasms, basic fibroblast growth factor (bFGF) is produced by thyroid carcinoma tumor cells (Daa *et al.* 1993, Kodama *et al.* 1994, Shingu *et al.* 1994, 1998, Eggo *et al.* 1995) and stromal cells (Shingu *et al.* 1998). Thyroid carcinoma cells also express FGF receptor-1 (Shingu *et al.* 1998) and bFGF stimulates their proliferation *in vitro* (Daa *et al.* 1993). These results indicate that bFGF plays an important role in the development of papillary carcinoma of the thyroid via an autocrine/paracrine loop (Daa *et al.* 1993). Moreover, epidermal growth factor (EGF) and its receptor EGF-R were widely expressed in normal thyroid and in all thyroid neoplasms (Makinen *et al.* 1988, van der Laan *et al.* 1995). The EGF-R immunostaining is stronger in neoplastic cells than in adjacent normal thyroid tissue (Westermark *et al.* 1996). Differentiated thyroid cancers bind more EGF than normal thyroid tissue and EGF stimulates the growth and invasion of differentiated thyroid cancer cells in culture and in nude mice (Hoelting *et al.* 1994). In papillary thyroid carcinomas, cytoplasmic EGF-R immunostaining has been demonstrated to be an independent indicator of tumor recurrence (Akslen *et al.* 1993, Akslen & Varhaug 1995), suggesting it may play a role in the pathogenesis of human thyroid carcinoma (Kanamori *et al.* 1989). Finally, the insulin-like growth factor (IGF)/IGF-receptor pathway also plays an important role in the pathogenesis of thyroid cancer. Thyroid carcinoma cells express the IGF-I receptor and IGF-I is produced locally in thyroid tumors by stromal cells in amounts significantly higher than in normal tissue (Vella *et al.* 2001). bFGF, EGF and IGF-I have been demonstrated to activate the phosphatidylinositol 3-kinase 3-kinase (PI3K)/Akt kinase pathway *in vitro* (Chen *et al.* 2000, Poulaki *et al.* 2002b, Andl *et al.* 2003). Akt overactivation has been reported in thyroid neoplasms (Ringel *et al.* 2001) and has been shown to promote resistance to apoptosis (De Vita *et al.* 2000). Stimulation of thyroid carcinoma cell lines with IGF-I *in vitro* upregulates expression of the apoptosis inhibitors FLIP, cIAP2 and XIAP; down-regulates the pro-apoptotic Bax; increases proliferation (Saito *et al.* 2001); and decreases sensitivity to TRAIL in a PI3K/Akt-dependent manner without affecting TRAIL receptor levels (Poulaki *et al.* 2002b). bFGF and EGF also have protective effects against TRAIL-induced apoptosis in thyroid carcinomas (Poulaki *et al.* 2002b). These observations provide another mechanism of functional resistance to death receptor-induced apoptosis, i.e. increased survival of the tumor cells *in vivo* due to paracrine/autocrine loops in the absence of direct genetic alterations of apoptosis-related genes in the tumor cells themselves.

Understanding the role of the microenvironment on tumor cell apoptosis and response to therapy suggested that targeting both the neoplastic cell and the microenvironment would provide a better anticancer effect and thus should be the basis for novel therapies. For example, the ansamycin antibiotic geldanamycin, which inhibits the chaperoning activity of heat shock protein 90 (Basso *et al.* 1993), has been demonstrated to activate the phosphatidylinositol 3-kinase (PI3K)/Akt kinase pathway *in vitro* (Chen *et al.* 2000, Poulaki *et al.* 2002b, Andl *et al.* 2003). Akt overactivation has been reported in thyroid neoplasms (Ringel *et al.* 2001) and has been shown to promote resistance to apoptosis (De Vita *et al.* 2000). Stimulation of thyroid carcinoma cell lines with IGF-I *in vitro* upregulates expression of the apoptosis inhibitors FLIP, cIAP2 and XIAP; down-regulates the pro-apoptotic Bax; increases proliferation (Saito *et al.* 2001); and decreases sensitivity to TRAIL in a PI3K/Akt-dependent manner without affecting TRAIL receptor levels (Poulaki *et al.* 2002b). bFGF and EGF also have protective effects against TRAIL-induced apoptosis in thyroid carcinomas (Poulaki *et al.* 2002b). These observations provide another mechanism of functional resistance to death receptor-induced apoptosis, i.e. increased survival of the tumor cells *in vivo* due to paracrine/autocrine loops in the absence of direct genetic alterations of apoptosis-related genes in the tumor cells themselves.

**Conclusions: future directions and therapeutic opportunities**

In thyroid cancer, the restoration of a pro-apoptotic state in neoplastic cells would enhance the effectiveness of...
anticancer therapy and the host’s own immune surveillance. Systemic administration of TNF-α or FasL is unlikely to be used therapeutically in the treatment of thyroid cancer, due to low, if any, antitumor activity and unacceptable toxicity. However, the discovery of the specificity of the anti-neoplastic action of TRAIL/Apo2L has led to a very promising candidate therapeutic reagent. A TRAIL-mimicking antibody, which binds to and activates TRAIL-R1, is currently being evaluated in phase 1 clinical trials in solid and hematological malignancies. Furthermore, novel therapies such as the geldanamycin analogs, which target locally produced survival factors and their signaling pathway, such as that of IGF-I, could exert increased antineoplastic activity clinically by targeting both the tumor cells and their microenvironment.

Yet, several questions still remain. For example, the mechanism of resistance of normal cells to TRAIL/Apo2L is still unknown, as several studies in multiple models have clearly dismissed the role of the ‘decoy’ receptors. So why is TRAIL so selectively potent against cancer cells? The fact that thyroid carcinoma cells have become sensitive to an apoptotic agent that spared their non-malignant precursors is difficult to explain and the only likely hypothesis is that sensitivity to TRAIL/Apo2L is strongly linked to an indispensable part of the neoplastic phenotype. The recent involvement of cell cycle regulation in the modulation of TRAIL/Apo2L sensitivity supports this hypothesis (Jin & El-Deiry 2001).

Finally, medullary thyroid carcinoma cells, in agreement with their different histogenetic origin, exhibit increased resistance to death-receptor induced apoptosis, even in the presence of sensitizing cytokines. This suggests a different regulation of apoptotic pathways in these cells and correlates with their resistance to chemotherapeutic agents in the clinical setting. Considering the poor clinical outcome of metastatic medullary carcinoma, the investigation of the apoptotic defect in these tumor cells is particularly important, as it could lead to urgently needed novel therapies.

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